Synopsis

Efficacy of probiotic bacteria in alleviating T helper cell-associated immune response

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Submitted for
For the award of the degree,
Doctor of Philosophy
in
Biotechnology

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July,, 2022

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Using inflammatory bowel disease as an *in vivo* model, the researchers hope to assess the efficacy of potential probiotic *Bacillus* and Bifidobacteria. Initially, an *in vitro* analysis using animal cell culture was performed, and then *in vivo* studies were investigated based on the results.

Man has accepted western routines as a result of massive changes in ecological situations and the industrial revolution, resulting in inflammatory responses, chronic infections, and psycho-emotional stress (Bosma-Den et al., 2012). Inflammation is necessary to fight infection; however, inflammatory mechanisms also underpin regular physiological events in the healthy body, such as embryonic implantation, fetal development, and tissue repair (Robinson et al., 2016). In some cases, inflammation in the intestine increases cell destruction, leading to diseases such as inflammatory bowel disease (IBD), inflammatory bowel syndrome (IBS), and others. IBD is exemplified by Crohn's disease and ulcerative colitis, both of which are chronic relapsing gastrointestinal disorders. IBD is a progressive and destructive disease that can result in stenoses, abscesses, fistulas, extraintestinal manifestations, colitis-associated neoplasias, and cancer (Neurath et al., 2002).

IBD is a chronic condition characterized by inflammation of the intestinal mucosa and, in some cases, deeper layers of the intestinal wall. T-helper cells, such as Th1 and Th2, produce inflammatory cytokines and defend the host against pathogenic microbes, tissue destruction, and inflammation (Plaza-Diaz et al., 2014). Crohn's disease is predominantly a T helper cell 1-driven immune response, characterized initially by increased interferon (IFN)-γ expression, followed by IL-6 and tumor necrosis factor (TNF)-α. Ulcerative colitis is believed to be a T helper 2 cell immune response, leading to increased production of pro-inflammatory cytokines, including IL-4 and IL-5 (Ramos & Papadakis, 2019). Few studies indicated that enhanced immune system abnormalities, normal gut flora, and environmental influences might play central roles in IBD (Peng et al., 2001). In the recent past, significant interest has been increased in probiotic cultures. Probiotic results in managing lifestyle diseases have been defensible through their capability to conserve stable intestinal microflora, avoid colonization by pathogens, and modify immune function (Wolvers et al., 2010).

Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host' (Hill et al., 2014). Probiotics are believed to stimulate the growth and well-being of the host and favorably modify the intestine ecology (Veizaj-Delia et al., 2010). Different probiotic species have been shown in animal models, such as rodents, to prevent or reduce gut inflammation when administered prior to inducing intestinal injury (Mishra et al., 2015). Interactions between probiotic strains and host epithelial cells would improve gut homeostasis, which is an important feature (Behnsen et al., 2013). By adhering to the epithelial layer and releasing pro and anti-inflammatory cytokines, many *Lactobacillus* and *Bifidobacterium* strains have demonstrated health benefits (Foligne et al., 2007). However, probiotic action on the host is highly strain-specific during immunomodulation (Devi et al., 2018).

Bifidobacteria are one of the most prevalent bacterial populations in human guts (Hidalgo-Cantabrana et al., 2018). They are thought to colonize the newborn's intestines upon birth and other bacteria from the mother's feces, vagina, and surroundings (Achi & Halami, 2017). Mother's milk also serves as an inoculum for the growth of bifidobacteria in the stomachs of breastfed neonates (Ventura et al., 2012). The most often used cell lines for studying the effects of bifidobacteria on the intestinal epithelium are HT29 and Caco-2. Although the immunological response of epithelial cells is limited in contrast to that of specialist immune cells, they are assumed to initiate interactions since they are directly exposed to the intestinal environment (Ruiz et al., 2017).

Probiotics based on *Bacillus* have been shown to improve growth, nutrient digestibility, humoral and cell-mediated immune responses, and the microbial ecosystem (Alexopoulos et al., 2004; Baker et al., 2013). *Bacillus licheniformis* (*B. licheniformis*) MCC 2514 is a native isolate isolated from sheep milk that has shown primary probiotic characteristics such as the ability to survive in gastric conditions such as low pH and the presence of trypsin, pepsin, and pancreatin. It was also antioxidant, antimicrobial, cell hydrophobic, and auto-aggregating (Shobharani & Halami, 2014). *Bifidobacterium breve* (*Bf. breve*) NCIM 5671 is a native isolate derived from a newborn fecal sample that contains phytase activity, milk fermentation capacity, antioxidant activity, and antibacterial activity (Achi & Halami, 2019).

Bf. infantis has been shown to reduce the colitis caused by TNBS (Javed et al., 2016). Researchers observed a decrease in symptoms and reduced injury to the mucosal construction in the group of colitis-affected rats supplemented with *Bf. infantis*, which suggested the preventive

role of probiotics (Zuo et al., 2014). The beneficial benefits of *B. cereus* HMPM18123 in a colitis mouse model, where it was discovered to relieve colitis symptoms. It has the potential to upregulate tight junction proteins, hence reducing damage to the intestinal epithelial barrier (Dokladny et al., 2016). *B. cereus* was discovered to regulate macrophage polarisation and suppress the TLR4-NFkB-NLRP3 inflammasome signaling pathways, resulting in anti-inflammatory actions. In addition, the injured gut microbiota was restored (Sheng et al., 2021).

Bacillus sp. was never widely recognized as a helpful microbe since several poisonous and pathogenic family members, such as *B. cereus, B. weihenstephanensis, B. anthracis,* and *B. thuringiensis*, were toxic and pathogenic (Elshaghabee et al., 2017). Most studies on *Bacillus* spp. focused solely on industrial applications, with relatively few on food items, where *Bacillus* exposure to probiotic features has not been adequately investigated. Understanding the probiotic property of *B. licheniformis* might open up new avenues for *Bacillus* use as a probiotic bacterium. A combination of facultative anaerobic *B. licheniformis* and anaerobic *Bf. breve* may be a more effective pathogen competitor in the gastrointestinal system. Bifidobacteria colonize the gut system early in childhood, becoming a permanent member of the GIT and bacilli a non-member.

Thus, the present study will contribute to understanding the role of *Bacillus licheniformis* MCC2514 and *Bifidobacterium breve* NCIM 5671, and both the culture in combination in reducing Th1 cell-mediated Crohn's disease and Th2 cell-mediated ulcerative colitis, which decreases inflammatory bowel disease. A combination of facultative anaerobic *B. licheniformis* and anaerobic *Bf. breve* can be a better competitor for the pathogen in the gut system.

To achieve the above goal, the below objectives were formed as a part of the investigation.

Objectives:

1. **To evaluate the mechanism of immune response of probiotic *Bacillus* and Bifidobacteria:** To understand the mechanism of the immune response, the adhesion and inflammation studies were performed, and those are interpreted in subchapters 2a and 2b, respectively.

2. **To study the efficiency of probiotic bacteria in reducing Th1 and Th2 cell response in *in vivo* model:** In order to evaluate Th1 and Th2 cell response, NaOH and TNBS induced inflammation in rats was analyzed and interpreted in subsections 3a and 3b, respectively.

3. **Development of probiotic food product using probiotic culture:** The product was developed using probiotic culture in this objective and explained in section 4.

Chapter 1: Introduction and review of literature

In this chapter, a brief introduction about the topic and the scope of the bacteria used have been described. In the second subchapter, the descriptive literature was provided on the topic of discussion. Initially, the history of the probiotic and its role in immunomodulation was discussed. Later, the health benefits and probiotics in disease conditions are explained. Further, the *Bacillus* and bifidobacateria's role as probiotic bacteria, both bacteria in the food product, *in-vitro* and *in-vivo* literature, were analyzed. In the later part, the types of inflammation, spectrum, and microorganism in inflammation were discussed. The inflammatory bowel disease, types, bacterial effect on IBD, and role of *Bacillus* and bifidobacteria in IBD treatment were discussed. Finally, the discussion was made on functional food such as dried probiotics and carrot products.

Chapter 2a: Adhesion efficiency of potential probiotic bacteria on HT-29 cell lines

The purpose of the study was to evaluate the colonization effectiveness of probiotic *B. licheniformis* and *Bf. breve* on the HT-29 cell line. *B. licheniformis* adheres to fibronectin at a rate of around 54.28 %, while *Bf. breve* adheres to mucin at a rate of about 70.23 %. *B. licheniformis* had an adhesion score of 138.85, whereas *Bf. breve* had a value of 43.05. In the adhesion percentage investigation, *B. licheniformis* adhered to 75.5 %, more than *Bf. breve*, which adhered to 32.66 %. However, the adhesion of *B. licheniformis* was greater than that of *Bf. breve*. Both bacteria considerably decreased the colonization of the pathogen *Kocuria rhizophila* in the invasion experiment by around 97.32 %, 97.87 %, and 82.19 % in the competitive assay, exclusion assay, and displacement assay, respectively. The cytotoxicity of the test bacterial strains against the HT-29 cell line was assessed using the MTT assay, with no loss of vitality detected in the

treated cells. As a result, this investigation showed that *B. licheniformis* and *Bf. breve* had excellent invasion on pathogen and adhesion capacity on HT-29 cell line.

Chapter 2b: Combined Effect of potential probiotics towards anti-inflammatory activity on HT-29 cell lines

The study aims to explore immunomodulatory gene expression on the HT-29 cell line in a single culture and combination of *B. licheniformis* and *Bf. breve*. In response to LPS-induced inflammation, the mixture of bacteria decreased the pro-inflammatory cytokines IL-1 (13.4), IL-12 (14.6), IL-8 (2.6), and IL-6 (1.9) while increasing TNF-α (21.2). Anti-inflammatory genes such as IL-4 (0.6), IL-10 (2.9), TGF-2 (92.2), and TGF-3 (85.8) folds, on the other hand, were upregulated. The combination of bacteria against oxidative stress decreased pro-inflammatory cytokines such as IL-1 and IL-6, IL-8, IL-12, and IL-18 while increasing anti-inflammatory cytokines such as IL-10, IL-4, TGF-2, and TGF-3. Pro-inflammatory cytokines were upregulated following the introduction of *K. rhizophila*. When *B. licheniformis* and *Bf. breve* was supplemented, the upregulated pro-inflammatory cytokines were reduced, but anti-inflammatory cytokines such as IL-4 (6.2), IL-10 (23.5), TGF-2 (166), and TGF-3 (28.4 times) were raised. When probiotic bacteria were introduced, gene expression of Toll-like receptor-2 increased roughly 26 times. Interferon-ELISA findings revealed that the expression was more significant when both bacteria were introduced together (7.19 ng/mL). When prospective probiotic bacteria were utilized in combination, they demonstrated more anti-inflammatory efficacy than a single culture. According to the findings, combining of aerobic *B. licheniformis* and anaerobic *Bf. breve* has anti-inflammatory action and can maintain an optimal gut environment during pathogen invasion and inflammation.

Chapter 3a: Effect of probiotic bacteria on NaOH-induced Crohn's disease in Wistar rats

This study looks at the prophylactic mechanism of probiotics tested in disease-induced settings. The inflammation was discovered in the bowel, namely the ileum and colon. CRP assessment revealed that NaOH-treated rats had 11% more inflammation than control rats, whereas probiotic-fed rats had 13% lower CRP levels. The antioxidant activity of the bacterial-treated rats was

superior to that of the NaOH-treated animals. Approximately 14% of antioxidant enzymes, such as SOD, were activated by probiotic bacteria therapy. At the same time, SOD levels in disease-induced treated rats were limited to 217.6 8.40 U/L. NaOH treatment activated the transcription factor (T-bet) of the Th1-driven immune response. The NaOH-treated rat increased T-bet gene expression by around 19.99 times, while probiotic bacteria reduced it by about 7.68 fold for *B. licheniformis* and 7.60 fold for *Bf. breve*. The NaOH treatment generated Th1-driven cytokines such as TNF-, IFN-, and IL-6. When the Spleen and Liver were treated with NaOH, inflammation was also identified. The treatment with the putative probiotic bacteria lowered the expression of antioxidant genes such as GPx and PON1. TNF- and IL-6 expression was decreased, whereas IL-10, an anti-inflammatory cytokine expression, was increased 3.38 fold in *B. licheniformis* and 3.43 fold in *Bf. breve*. This study found that NaOH produced a Th1-driven immune response, resulting in Crohn's disease and that probiotic bacteria provided an anti-inflammatory response and reduced the severity of disease development.

Chapter 3b: Efficiency of probiotic bacteria on TNBS-induced ulcerative colitis

In addition to the NaOH-induced study, *B. licheniformis* and *Bf. breve* was used as an immune modulator in the TNBS-induced ulcerative colitis investigation. TNBS produces transmural inflammation with morphological and histological characteristics in the stomach and increases cell-mediated immune responses. This study aims to assess the probiotic efficacy of TNBS-induced ulcerative colitis in Wistar rats. The tumor-like formation was discovered in the colons of TNBS-inflamed rats. Compared to TNBS treatment, the combination of probiotic bacteria reduced nitric oxide generation by 65.2 percent. C-reactive protein synthesis was reduced by around 12% and 10.8%, respectively when *B. licheniformis* and *Bf. breve* was used in comparison to TNBS-treated rats. Liver damage was observed in the TNBS-treated rats as found in SGPT and SGOT assays. These enzymes decreased to 75.4% for SGPT and 42.5% for SGOT analysis on the addition of probiotic bacteria in combination compared to TNBS treated rats. A significant deviation was observed in Superoxide dismutase in all sets of rats fed with probiotics. The transcriptional factor responsible for Th2 cell immune response (GATA3) was analyzed, and the elevation in the gene expression was found to be about 5.31 folds. The FOXP-3 responsible for T-regulatory cells was expressed about 0.91 folds upon the treatment with the combination of bacteria. The gene

expression of antioxidant enzymes such as iNOS has been reduced by about 1.11 folds. During the bacterium treatment, cytokines unique to the Th2-driven immune response, such as IL-4, IL-5, and TNF-α, were induced. The *B. licheniformis* and *Bf. breve* suppressed the Th2-driven immune response.

Chapter 4: Osmo-dried probiotic infused carrot product formulation and analysis

The carrot product in this study was created by incorporating probiotic *B. licheniformis* into carrot shreds and drying them at 60 °C. After drying for 6-8 hours, the moisture content was reduced to 8-10%. Probiotic-infused Osmo dried carrot shreds had a water activity of around 0.639 0.001a_w, whereas un-blanched dry carrot shreds had a water activity of 0.473 0.005a_w. A little color variation was seen at 60.47 dE*ab. Total phenol decreased from 49.07 0.40 mg/GAE (probiotic-infused Osmo dry carrot shreds) to 97.78 1.06 mg/GAE (un-blanched dried carrot shreds). There was little difference in flavonoids between the control and the sample. The product's overall acceptability was 7.73 out of 10 based on sensory evaluation. The sample's proximate and minerals were examined. When polyphenols were detected using UPLC, Chlorogenic acid and the other five main polyphenols were discovered in both samples. HPLC was utilised to assess the -carotene (probiotic-infused Osmo dried carrot shreds 0.339 0.0mg/ mg extract and un-blanched dry carrot shreds 0.565 0.0 mg/ mg extract) and -carotene. Room temperature and viability were determined to be 7.23 0.02, 6.81 0.07, and 6.58 0.03 log CFU for PIODCS samples stored at -20°C and 4°C, respectively. Even after 20 weeks, the Bacillus' viability was maintained in the three distinct storage settings. The results of the investigation above indicate that *B. licheniformis* has outstanding viable stability when supplemented with Osmo dried carrot.

Chapter 5: Summary, conclusion, and future perspective

In this chapter, the overall summary of the results from all three objectives was listed pointwise, and the conclusion was given after the summary. The future perspective of the work is discussed pointwise.

The overall study suggested the potential probiotic *B. licheniformis* and *Bf. breve* has displayed excellent adhesion and invasion ability over pathogen. The combination of *B. licheniformis* and *Bf. breve* has demonstrated good anti-inflammatory gene expression. Both bacteria could be a promising candidates for reducing inflammatory symptoms and demonstrated excellent agents as an inducer of anti-inflammatory cytokines in in-vitro and in-vivo analyses. The *B. licheniformis* has been an excellent probiotic agent in the formulation of functional food. In future, *B. licheniformis* MCC 2514 and *Bf. breve* NCIM 5671 can be taken to the clinical study, which can be a prominent probiotic in the field of probiotic usage.

The bibliography of the literature referred to is mentioned below

Publication

1. **Rohith H.S.,** & Halami, P. M. (2021). The combined effect of potential probiotic *Bacillus licheniformis* MCC 2514 and *Bifidobacterium breve* NCIM 5671 towards anti-inflammatory activity on HT-29 cell lines. Probiotics and Antimicrobial Proteins, 1-12. https://doi.org/10.1007/s12602-02109851-y

2. **Rohith, H. S.,** & Halami, P. M. (2021). *In vitro* validation studies for adhesion factor and adhesion efficiency of probiotic *Bacillus licheniformis* MCC 2514 and *Bifidobacterium breve* NCIM 5671 on HT-29 cell lines. Archives of Microbiology, 1-10. https://doi.org/10.1007/s00203-02102257-y

Abstract in the conference

1. **Rohith H.S.,** and Halami PM. Immunomodulatory effect of *Bacillus licheniformis* MCC 2514 and *Bifidobacterium breve* NCIM 5671 on HT-29 cell line. 5th Biennial Conference of Probiotics Association of India & International Symposium on Probiotics & Immunity: Way forward to Microbial Therapy. November 19-20, 2020.

2. **Rohith H. S.**, Akash-Kumar Bal and Halami PM. The efficiency of *Bacillus* spp. producing substances like subtilin and bacteriocin against food-borne pathogens, drug-resistant bacteria, and fungi. Abstract No. FBFM 026 of 8th IFCON-2018 CSIR-CFTRI, Mysore; Dec. 12-15, 2018.

General article

1**. Rohith HS** and Halami PM. 2017. Probiotic effectiveness for inflammatory bowel disease in the News Letter, Probiotic Association of India. Vol 13, Page 12-16.

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